SEROLOGICAL RELATIONSHIPS OF TYPE-SPECIFIC AND DEGRADED PNEUMOCOCCI.

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In a previous paper (1) it was shown that a strain of Pneumococcus Type I loses its specificity and virulence when subjected to certain unfavorable cultural environments. Yoshioka (2) has brought about a similar process of degradation in strains of Pneumococcus Types II and III. From these studies it is apparent that at least two general and widely different forms exist among pneumococci of the fixed types (Types I, II, and III), one comprising the virulent, type-specific, so called S strains, and the other including the avirulent, non-specific, so called R strains.

Both Griffith (3) and Yoshioka found that antisera prepared by using R strains for immunization did not agglutinate the virulent type-specific organisms and did not protect against infection caused by them. On the other hand, R strains were agglutinated by R sera and also by type-specific antipneumococcus sera regardless of type.

Type-specific pneumococci are capsulated and capable of elaborating the type-specific soluble substances which have been identified by Avery and Heidelberger as the carbohydrate constituent of the pneumococcus cell (4). Pneumococci of the R forms are not capsulated and do not elaborate the soluble substances which endow the cell with type specificity. In sequence with the recent observations of Avery and his associates (5–7), on the antigenic properties of the cellular constituents of pneumococcus, it seemed of interest to study further the immunological relationships between the so called S and R forms of pneumococci.

Methods.

Preparation of Sera.—Type I, II, and III pneumococci were repeatedly grown in broth containing dilutions of immune serum of the respective type, until upon

plating the cultures only colonies of the rough, avirulent R form appeared. From ten to twenty transfers in 1 per cent immune sera were necessary. Antipneumococcus sera were then prepared with the three type-specific S strains and were called Type I, II, and III S sera, and also with the variant R strains of their respective types which were designated Type I, II, and III R sera.

For the preparation of immune sera, the bacteria from young, actively growing cultures were suspended in salt solution and immediately killed at 56°C. to minimize the amount of autolysis or cell solution. Autolysis is undesirable since Avery and Heidelberger (7) have found that dissolution of the bacterial bodies is accompanied by dissociation of the specific antigenic complex and consequent loss of power to provoke type-specific antibodies. Rabbits were immunized according to the method of Cole and Moore (8) by the intravenous injection of pneumococci of the three specific types and of the variant strains derived from them by methods already described.

Sera were absorbed by mixing 10 per cent dilutions of the homologous serum with definite volumes of pneumococcus bodies obtained by centrifuging heated broth cultures. For instance, 0.2 cc. of the bacterial sediment was emulsified in 2 cc. of the diluted serum. The mixture was incubated for 2 hours at 37°C. and placed in the ice box overnight. The bacteria were then removed from the serum by centrifuging. R sera usually required a second exposure with one-twentieth volume of pneumococci for complete absorption.

Autolysates of the pneumococci were obtained by suspending the bacterial sediment from 150 cc. of an 8 hour dextrose broth culture in 10 cc. of salt solution and allowing the cells to disintegrate at 37°C. for 24 to 48 hours or until autolysis was nearly complete. The centrifuged supernatant fluids or autolysates were faintly opalescent. Solutions of pneumococci in bile did not give clear-cut reactions and were not used.

The pneumococcus protein and the anti pneumococcus-protein serum were prepared according to the methods of Avery and Heidelberger (4) and Avery and Morgan (5).

Considerable difficulty was encountered owing to the rapid autolysis and spontaneous agglutination of the II R and III R strains when used for agglutination tests. This was overcome to a large extent by transferring the strains eight or ten times in plain broth and finally killing by heat. Suspensions of heat-killed pneumococci were used in all agglutination tests.

Experiments with Antipneumococcus Serum.—The antipneumococcus serum (S) prepared by immunization with intact cells of the type-specific, virulent form contains predominantly an antibody reactive only with the soluble specific substance of the homologous type. However, since cultures may contain both intact cells and a variable amount of degraded cells as well as products of cell dissolution (7), and since in the material used for immunization dissociation is

TABLE I.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum.

Agglutination.					Precipitation.		
Bacterial suspensions.	Antipneumococcus serum (S) (Type I).			Normal rabbit serum.	Bacterial solutions.	Antipneumococcus serum (S) (Type I). 1:10	Control normal rabbit serum. 1:10
	1:10	1:20	1:40	1:10			
Type I–S pneumococci.....	++++	+++	+	−	Type I specific substance 1:40,000.....	++++	−
" II–S "	−	−	−	−	Type I–R autolysate.....	++	−
" I–R "	++	+	+	−	" II–R "	++	−
" II–R "	+	+	+	−	" III–R "	++	−
" III–R "	++	+	+	−	Pneumococcus protein solution 1:300.....	++	−
					Control salt solution.....	−	−

++++ = firm disc.
+++ = disc easily broken up.
++ = coarse agglutination or heavy precipitate.
+ = fine agglutination or light precipitate.
− = no agglutination or precipitation.

bound to occur in spite of precautions to prevent it, some of the cell protein becomes free and acting independently of the antigenic complex of the intact cell gives rise to an antiprotein immune body which is species-specific and distinct from the type-specific antibody. The usual antipneumococcus sera apparently contain both forms of antibodies.

It has been found on previous occasions that all R strains as well as the protein of pneumococci react in immune S sera regardless of type derivation. On the other hand, type-specific pneumococci (S forms) agglutinate only in their corresponding type sera. Agglutination and precipitin reactions of Type I-S antipneumococcus serum with suspensions and autolysates of various pneumococci are shown in Table I.

In Table I evidence is presented that Type I antipneumococcus serum contains specific agglutinins for homologous S strains, and precipitins for the homologous specific substance. It is not reactive with pneumococci of heterologous specific types. However, in addition to the type-specific antibodies there are present agglutinins for cells of the R forms, regardless of type derivation, and precipitins which react with autolysates of R strains and with solutions of pneumococcus protein. It would seem, therefore, that antipneumococcus serum contains the type-specific antibody together with antibodies which are common to all R strains and pneumococcus protein.

If antipneumococcus serum of this nature is absorbed with organisms of a homologous S type, it is possible to remove the type-specific antibody and leave the protein antibody intact. Table II shows the reactions obtained with a serum so treated.

It is apparent that neither the homologous S strain nor soluble specific substance derived therefrom is precipitated by the absorbed serum. The agglutinins and precipitins common to the R strains, however, remain, although somewhat diminished in titre. It is assumed that the diminution in titre is due to the absorption of some of the non-specific antibodies by some degraded forms or products of autolyzed pneumococci which appeared in the S culture during the preparation of the suspension for absorbing.

Just as it is possible to absorb the type-specific antibody from antipneumococcus serum and leave the protein antibody, so it is

likewise possible to absorb the latter and leave the former antibody practically undiminished in titre. Table III shows the serological

TABLE II.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum Absorbed with Type I-S Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I-S pneumococci.....	−	−	−	Type I specific substance 1:40,000.....	−
" I-R "	+	+	+	Type I-R autolysate.....	+
" II-R "	+	+	−	" II-R "	+
				" III-R "	+
				Pneumococcus protein solution 1:300.....	+
				Control salt solution.....	−

TABLE III.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum Absorbed with Type I-R Pneumococcus.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I-S pneumococci.....	++++	+++	+	Type I specific substance 1:40,000.....	++++
" I-R "	−	−	−	Type I-R autolysate.....	−
" II-R "	−	−	−	" II-R "	−
" III-R "	−	−	−	" III-R "	−
				Pneumococcus protein solution 1:300.....	−

behavior of a type-specific serum absorbed with an R strain of pneumococcus.

Absorption of Type I antipneumococcus serum with a strain of

the degraded R forms derived from the same type leaves the titre of the type-specific antibody unaffected. Both the agglutinins for the R forms of pneumococci and the precipitins for autolysates and pneumococcus protein are completely removed, leaving a serum reactive only with homologous Type I-S pneumococci and soluble specific substance derived from them.

The experiments cited above have been repeated and results similar to those for Type I serum have been obtained with antipneumococcus sera and the corresponding organisms of Types II and III.

Several attempts were made to produce a serum *in vivo* containing only the type-specific antibody. The usual procedure of immunization was employed with the exception that for each injection organisms were obtained directly from an infected mouse. The mouse was inoculated intraperitoneally with pneumococci and killed in 12 hours. The peritoneal cavity was washed with saline and the suspension immediately centrifuged, washed once, and heated at 56° for ½ hour. By this rapid procedure it was hoped that autolysis with its coincident dissociation of the carbohydrate and protein constituents of the bacterial antigen would be eliminated. However, after immunization of six rabbits by this method it was found that each serum contained varying traces of the non-specific protein antibody, although but slight in amount when compared with antipneumococcus sera prepared in the usual way.

Experiments with Anti-R Pneumococcus Serum.—Since no type-specific substances are elaborated by the R strains it seemed likely that sera prepared against these strains would not contain any of the specific antibodies, but only those reacting in common with all R strains. Agglutination and precipitin reactions against specific and degraded strains of pneumococci by an anti-R serum are shown in Table IV.

It is evident from Table IV that the anti-R serum does not contain any type-specific agglutinins for the S strain from which the R form was derived. Neither does it contain precipitins for the homologous soluble specific substance. Agglutinins are present, however, for both homologous and heterologous R strains. There are also precipitins for pneumococcus autolysates of all types, both S and R. The autolysates of the S strains obviously contain the free specific element

and the free protein, but the latter alone is reactive in R serum. By virtue of its common nature, the protein of each autolysate is therefore

TABLE IV.

Agglutination and Precipitin Reactions with Type I-R Serum.

Agglutination.				Precipitation.	
Bacterial suspensions.	Serum dilutions.			Bacterial solutions.	Serum dilution. 1:10
	1:10	1:20	1:40		
Type I–S pneumococci.....	−	−	−	Type I specific substance 1:40,000.....	−
" I–R "	++	+	+	Type I–S autolysate.....	+
" II–R "	++	+	+	" II–S "	+
" III–R "	++	+	+	" III–S "	+
				" I–R "	+
				" II–R "	+
				" III–R "	+
				Pneumococcus protein solution 1:300.....	+

TABLE V.

Agglutinanon and Precipitin Reactions with Type I-R Serum Absorbed with Type I-R Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilutions. 1:10
	1:10	1:20	1:40		
Type I–R pneumococci.....	−	−	−	Type I–S autolysate.....	−
" II–R "	−	−	−	" II–S "	−
" III–R "	−	−	−	" III–S "	−
				" I–R "	−
				" II–R "	−
				" III–R "	−
				Pneumococcus protein.....	−

reactive. Similarly, pneumococcus protein, regardless of the form from which it is isolated, is precipitated by the R serum.

It is possible to absorb the antibodies from an R serum with an R

strain. This procedure removes the agglutinins for the R strains and the precipitins for the pneumococcus protein, leaving the serum inactive. The agglutination and precipitin reactions of a Type I-R serum absorbed with a Type I-R strain are given in Table V. The absorbed serum being inactive does not agglutinate suspensions of the R organisms or precipitate pneumococcus protein either in its purified isolated form or as it occurs in cell autolysates.

Protocols similar to Table IV were obtained by using II-R and III-R sera. Reactions similar to those shown in Table V were obtained

TABLE VI.

Agglutination and Precipitin Reactions with Anti Pneumococcus-Protein Serum.

Agglutination.				Precipitation.	
Bacterial suspensions.	Serum dilutions. Anti pneumococcus-protein serum. 1:10	1:20	1:40	Bacterial solutions.	Serum dilution. 1:10
Type I–S pneumococci.....	−	−	−	Type I specific substance 1:40,000.....	−
" I–R "	++	+	+	Type I–S autolysate.....	+
" II–R "	+	+	+	" II–S "	+
" III–R "	+	+	+	" III–S "	+
				" I–R "	+
				" II–R "	+
				" III–R "	+
				Pneumococcus protein solution 1:300.....	+

with a III-R serum absorbed with a II-R strain, and with a I-R serum absorbed with a III-R strain of pneumococci.

Experiments with Anti Pneumococcus-Protein Serum.—Because of the consistent similarity in precipitin reactions of the pneumococcus autolysates, and of the prepared pneumococcus protein in the foregoing experiments, it seemed of interest to observe the serologic behavior of a serum prepared against this protein. The agglutination and precipitin reactions of an anti pneumococcus-protein serum with S and R strains are shown in Table VI.

It is significant that the reactions in Table VI resemble those shown in Table IV. Apparently sera prepared with R strains and with pneumococcus protein have similar serologic properties. The antiprotein element in the serum obviously being common to the protein of all types of pneumococci, both S and R, causes a precipitate in all of the autolysates and agglutinates suspensions of all R strains. The intact S organisms are not affected.

The similarity between the antiprotein serum and the anti-R serum is further demonstrated by the absorption of the agglutinins in the antiprotein serum with R strains. The results are tabulated

TABLE VII.

Agglutination and Precipitin Reactions with Anti Pneumococcus-Protein Serum Absorbed with Type I-R Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I–R pneumococci.....	—	—	—	Type I–S autolysate.....	—
" II–R "	—	—	—	" II–S "	—
" III–R "	—	—	—	" III–S "	—
				" I–R "	—
				" II–R "	—
				" III–R "	—
				Pneumococcus protein solution.....	—

in Table VII. The resemblance of Table VII to Table V is self-evident. The agglutinins for the R strains and the precipitins for the cell autolysates and pneumococcus protein have been removed, leaving an inactive serum.

DISCUSSION.

Avery and Neill (6) have shown that solutions of pneumococci have antigenic properties strikingly different from those of the intact cells themselves. When cell solutions are used for immunization, antibodies arise which react with the common protein alone. On the

other hand, when the uninjured bacterial cell is used for immunization, antibodies arise which react with the type-specific carbohydrate substance. By virtue of the common properties of the protein constituent of pneumococci, sera containing the antibody to this protein react with the protein from all types of pneumococci. Antiprotein sera obtained by immunization with dissolved pneumococci are similar in their serologic reactions to sera produced by the injection of the isolated protein.

The experiments recorded in the present paper show that sera prepared by immunization with pneumococci of degraded types (R strains) resemble in their reactions sera prepared by immunization with the cell protein alone. It would seem, therefore, that R pneumococci stimulate the formation of antibodies by virtue of the protein constituent of the cell body. Previous mention has been made of the absence of capsule formation and of the associated specific substance in the R strains. Consequently the R strains, devoid of the type-specific antigen, cannot stimulate the formation of antibodies reactive with the type-specific substances and therefore have only the antigenic properties of the isolated protein. Thus, on account of the common antigenic properties of the protein of all pneumococci it is not surprising that R sera cross-agglutinate with R strains regardless of type derivation, and precipitate the free protein in autolysates of S strains.

The results on the whole substantiate the deductions of Avery and Heidelberger (7) regarding the relationship of the carbohydrate and protein constituents of the pneumococcus cell.

Assuming that the loss in type specificity of an S strain after degradation to the R form is due to a loss in the capacity to elaborate the type-specific carbohydrate substance associated with the capsule, and that the R form is merely an S cell minus this factor, it is possible to interpret the immune mechanism involved according to the views of these authors. Applying these conclusions to the results of their study it is only necessary to substitute in their scheme (7, Fig. 1) the symbol for protein (P) by R representing an R cell. The type-specific cell would then be an S-R combination and the R cell, R alone.

The confusing cross-reactions occasionally encountered in typing

pneumococci with the usual diagnostic sera, and the cross-reactions occasionally noted in S cultures containing unusual numbers of degraded pneumococci, are thus in part explained.

CONCLUSIONS.

1. Immune sera prepared with the degraded or variant forms of pneumococci (R strains) are similar in their reactions to sera prepared with the protein or cell solutions of pneumococci. They contain antibodies reactive with the protein of all types of pneumococci, but no antibodies reactive with the type-specific substances.

2. Pneumococci of the variant or R form, regardless of type derivation, are serologically identical and have the antigenic characteristics of pneumococcus protein. They evoke the species-specific and not the type-specific antibodies.

3. Antipneumococcus sera obtained by immunization with S strains may contain species-specific antibodies in addition to those which are type-specific. Each kind of antibody can be removed separately from these sera by selective absorption with the R or S strains of pneumococci.

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